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Development of glucose biosensor based on ZnO nanoparticles film and glucose oxidase-immobilized eggshell membrane

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ABSTRACT

A novel electrochemical glucose biosensor was developed by depositing an ionic liquid (IL) (e.g., 1-ethyl-3-methylimidazolium trifluoromethanesulfonate; [EMIM][Otf]), ZnO nanoparticles (ZnONPs) and eggshell membrane (ESM) on a modified glassy carbon electrode (GCE) for determination of glucose. Glucose oxidase (GOx) was covalently immobilized on eggshell membrane with glutaraldehyde as a cross-linker. Methylene blue was used as a redox indicator to enhance the electron transfer capacity and to ensure stability of both the oxidized and reduced forms in the reaction of enzyme and substrate. The morphological characteristics of microstructures eggshell membranes, chitosan, GOx/ESM, GOx/ZnONPs/IL/ESM and GOx/ZnONPs-IL/CHIT were observed using scanning electron microscopy (SEM). The effects of scan rate, time and pH on the response of glucose biosensors were studied in detail. Under optimal conditions (pH 6.5, 50 s), cyclic voltammetry showed different glucose concentrations on the range of 1×10^{-12} to 0.6 M, with a detection limit of 1×10^{-13} M. The GOx/ZnONPs/IL/ESM was found to be more sensitive as compared to GOx/ZnONPs-IL/CHIT. This developed glucose biosensor detection approach has several advantages such as fast, simple and convenient method, sensitivity, low cost, eco-friendly, low concentrations and remarkable catalytic activities of current signals during glucose reaction.

1. Introduction

Discarded egg shells are a major waste product of the poultry processing industry. The current methods of disposal of egg shells which include microbial composting in landfills as well as incineration contribute to the release of carbon dioxide to the environment thus contributing to the carbon footprint of the industry [1]. Eggs are a primary source of cheap protein in many underdeveloped and developing Asian countries as compared to developed countries which rely on value added protein products for consumption. This leads to an increase in the amount of egg shell waste in developing countries [2]. Egg shell waste can be recycled, modified and processed in order to develop a matrix for the immobilization of enzymes. The egg shell membrane (ESM) is comprised of the egg white (albumen) and the inner surface of the egg shell. There are two membranes; thick outer membrane is attached to the shell and a thin inner membrane [3]. ESM is required for the formation of the eggshell and as a means to retain albumen, as well as curtail the passage of bacteria. Physically, ESM bears all the characteristics of connective tissue. It is relatively thin, colorless and has high structural strength as a result of its collagen content, protein fiber and embedded proteins. ESM exhibits high porosity, demonstrates antibacterial activity, is anti-inflammatory, and contains a high amount of minerals and amino acids. ESM has a large surface area and permits the diffusion of gas and water molecules. These properties make it an ideal, naturally occurring biological platform for enzyme immobilization [4,5]. Several modifications on glassy carbon electrodes have been done and results yield are promising. The use of chitosan (CHIT) film is one of the preferable nanocomposite membrane used for enzyme immobilization [6] due to its abundance, low cost, regenerability, zero toxicity, good gel-forming properties, amenability to pH manipulation and biodegradability [7–10]. Researcher has found that CHIT immobilized on glassy carbon electrode (GCE) resulted in a higher oxidation rate of tryptophan [11]. Both chitin and CHIT are functional chelating agents as they have higher percentage of nitrogen (6.89%) as compared to those of modified cellulose (1.25%). However, the percentage of nitrogen still depends on the degree of deacetylation [10].

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The new Freedonia industry study reported an approximately 6.3% increase in the world enzyme demand for the period 2011–2015, led by animal feed and food and beverage enzymes, which both benefited from the expansion of the middle class in rapidly developing economies. The world enzyme demand arose at a healthy pace to reach $7.6 billion in 2015. The Asia/Pacific region will also undergo a rapid increase in enzyme demand due to strength in China and India. By observing this situation, glucose oxidase (GOx) is an important enzyme that contributed to the growth of the industry.

Glucose oxidase has high demanded in many industrial applications and the demand for more stable, highly active and specific enzymes are growing rapidly [12]. Immobilization is the process of attaching enzyme onto insoluble material using physical or chemical interaction. Immobilization leads to the partition of the enzyme catalyst from the reaction mixture, and can increase enzyme turnover, which in turn leads to higher efficiencies and the associated reduction in operational costs. The immobilized enzyme can be used repeatedly and continuously. This resolves the issues associated with effluent disposal. In addition to this, immobilized enzymes typically have greater thermal and operational stability than the soluble versions of the enzyme.

Enzyme immobilization enables the bioprocessing industry to harness the full potential of a particular enzyme. Immobilization facilitates the separation of the enzyme catalyst from the reaction mixture, increases the efficiency of the enzymatic resulting in a lower processing cost. Biochemical studies have demonstrated that enzyme immobilization leads to a well-balanced overall performance, reasonable immobilization yields, low mass transfer limitations, and high operational stability [13]. The immobilization strategy is vital to maintain the biological activity of the enzyme [14]. Cross linking is one of the strategies which have been applied effectively to improve the stability and activity of an enzyme. For instance, biocompatibility between glutaraldehyde and eggshell membrane contributes to functional stability [15].

Two considerations have to be taken into account when developing electrochemical biosensors, Firstly, the sensor should demonstrate a high degree of sensitivity across its working range and secondly, the sensor should be simple to deploy and to operate.

Currently available methods for the detection of glucose such as SDS–PAGE, FTIR, spectrophotometry and scanning electron microscopy (SEM) [16] have been reported to be extremely sensitive and reliable but have inherent drawbacks which include complexity of sample preparation and specialized training for operation of the respective equipment. The size of these instruments and their high cost do not permit deployment in field testing laboratories. All of these factors have contributed to a great interest in developing enzyme biosensors based on immobilization of enzyme on modified electrode.

The application of nanoparticles (NPs) to biosensors is now considered to be an emerging area of innovation. Nanosensors have been developed for the specific detection of biological molecules, e.g., enzymes (proteins) [17] and nucleic acids [18] and as well as infectious agents [19]. Nanoparticles are able to promote efficient electron transfer between the electrodes and the active site of the enzyme due to their diminutive size and unique physical characteristics. Zinc oxide nanoparticles are one of the most important nanomaterials due to their unique electronic, metallic and structural characteristics. A significant number of publications of nanoparticle-sensing research have focused on the ability of surface-nanostructure ZnONPs to accelerate electron transfer reactions with electroactive species. Zinc oxide (ZnO) is a wide band-gap semiconductor and is garnering the attention of the scientific community for its application in the field of electrochemical biosensors due to its unique properties like high isoelectric point (IEP) and biocompatibility [20]. Owing to its high IEP the surface of ZnO matrix can adsorb the biocatalysts having low IEP (~4.2 for glucose oxidase) via electrostatic interaction. The possibility of growing large area thin films of ZnO nanostructures on variety of materials at a relatively low cost has led to a rapid progress in the development of ZnO-based biosensor [20–22]. It is confirmed that ZnO provides direct charge transfer for the immobilization of enzymes and retention of the biological activity on its surface [20].

Ionic liquids (ILs) are composed of large organic cations and a diversity of anions that exist in the liquid state. ILs have become the subject of intense scientific scrutiny due to their unique chemical and physical properties, such as high chemical and thermal stabilities, negligible vapor pressure, high ionic conductivity, low toxicity, and their ability to serve as solvents for a wide range of organic and inorganic compounds [23,24]. ILs have widely used as modifiers on electrode surfaces for fabrication of biosensors [25] and gas sensors [26] due to their unique electrochemical properties, such as high ionic conductivity and relatively wide electrochemical gap. ZnONPs-IL/ESM composite materials have a potential application in the development of electrochemical biosensors.

The objective of this study was to explore the possibility of utilizing egg shell membranes, in conjunction with nanoparticles and ionic liquids, as a platform for the immobilization of glucose with substrate. Incorporation of ionic liquid, ZnO nanoparticles, eggshell membrane and methylene blue were explored to increase the electrochemical signals with a redox indicator, and enhanced the sensitivity of glucose detection. The glucose concentrations were readily determined through measuring the current derived from the electrochemical reaction of glucose. The morphological characteristics of immobilized enzyme and nanomaterials onto the eggshell membrane were observed using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Apparatus and electrodes

The voltammetry measurements were carried out with a Metrohm AutoLab B.V (Ultrecht, The Netherlands) potentiostat/galvanostat using the software package NOVA 1.8. A Metrohm glassy carbon electrode (3 mm) was used as the electrode to be coated for the covalent immobilization of the enzyme and nanomaterials onto the eggshell membrane. An Ag|AgCl|KCl 3 M reference electrode and a platinum (Pt) wire counter electrode were also employed.

2.2. Reagents and solutions

The eggshell wastes were collected from the local restaurants in Kingfisher, Kota Kinabalu, Sabah, Malaysia. Zinc oxide nanoparticles, ionic liquid and methylene blue (MB) were purchased from Sigma (USA). Stock solution of MB (1 mM) was prepared in a 50 mM sodium phosphate buffer (pH 7.0, 5 mM) solution. The diluted solutions were prepared by appropriate dilution with the same buffer solution. The other solution was a 2.5% glutaraldehyde which was utilized as a crosslinking agent for immobilization of enzyme. All chemicals used in the experiments were of analytical-reagent grade. Deionized water was obtained from a Millipore Milli-Q purification system.

2.3. Preparation of eggshell membrane

Waste eggshells were incubated in 99.9% acetic acid at 4 °C for 24 h to obtain the whole egg shell membranes. The whole eggshell

membrane was carefully peeled from fresh weakened incubated eggshell. The obtained ESM was cut into two equal parts and cleaned with sterile distilled water while albumen and yolk had been removed. The cleaned ESM was directly used for enzyme immobilization or stored at 4 °C in phosphate buffer (pH 7.0, 5 mM) solution for further uses.

2.4. Preparation of the GOx/ZnONPs/[EMIM][Otf]/ESM modified electrode

Before modification, glassy carbon electrode (GCE) (3 mm-diameters) was polished with 0.05 μm alumina slurry. For optimum cleanliness it was wise to use gloves when handling the electrodes. This precaution was necessary for skin oils not contact onto the electrode. The electrode was polished using light pressure according to follow a figure '8' pattern for 1 min. The electrode was immediately rinsed with distilled water. After that, the electrode was sonicated with ultrasonic bath for 2 min to remove any physically adsorbed substances. Finally, the electrode was dried thoroughly using nitrogen gas.

Circular (radius nearly 2 cm) eggshell membrane was removed from the phosphate buffer. An appropriate amount of the ZnO nanoparticles were dispersed onto the eggshell membrane. It was sonicated for 20 min after stirring for 8 h. The mass ratio of ZnONPs:ESM was 1:5. A 20 μL of 100 mg/mL enzyme solution in a 5 mM phosphate buffer (pH 7.0) was added and membrane was kept for 90 min at 4 °C for adsorption. After adsorption, 10 μL of a 2.5% (w/w) glutaraldehyde solution as the crosslinking agent was dropped onto the surface of the membrane and stood for 5 min. After that, a glass rod was gently used to spread the glutaraldehyde solution thoroughly on the membrane surface. After 5 min, the membrane was immersed and washed with phosphate buffer (pH 7.0, 5 mM) solution for removing any unbounded substances. [EMIM][Otf] was dispersed in the ZnONPs/ESM composite, and then sonicated for 3 h to produce homogeneous suspension. The ratio of ionic liquid was fixed at 3.0% (v/v) in this experiment.

2.5. Preparation of the GOx/ZnONPs/([EMIM][OTF])/Chit

For immobilization of GOx with chitosan, GOx solution (100 mg/mL) was prepared by dissolving 6 μL of stock glucose solution (50 mg/mL) in 10 μL of 0.05 mol L^{-1} buffer (pH 7.0), which corresponded to the optimum pH. The GOx solution was thoroughly mixed with the chitosan in the volume ratio of 1:20 and sonicated for 15 min. Then, 10 μL of the mixture was deposited on the surface of the GCE and allowed to evaporate at room temperature. This procedure resulted in the fabrication of homogenously dispersed GOx/chitosan immobilized on the glassy carbon electrode surface. An appropriate amount of the ZnONPs nanoparticles were dispersed on the Chit. It was then sonicated for 20 min after stirring for 8 h. The mass ratio of ZnONPs:Chit was 1:5. A 20 μL of GOx solution in a buffer (pH 7.0, 0.05 mol L^{-1}) was added. An ionic liquid was dispersed in the ZnONPs/Chit composite, and then sonicated for 3 h to produce homogeneous suspension. The ratio of ionic liquid, [EMIM][Otf] was fixed at 3% (v/v) in the experiments.

2.6. Assembly of electrochemical biosensor and determination

Enzyme immobilized-egg shell membrane was positioned on the surface of a Thermo-Orion glassy carbon electrode and kept in a steady position by an O-ring. Then, the electrode was dried at room temperature for at least 5 min. The counter and reference electrodes were immersed together into a stirred reaction media containing phosphate buffer solution. An appropriate amount of glucose was injected into the reaction media and the concentration of the accumulation products signal was measured and processed by an AutoLab Nova 1.8 software package. The same protocol was applied onto the unmodified GCE to test the reactions of the enzyme with substrate.

2.7. Methylene blue accumulation onto the modified electrodes

Methylene blue (MB) was accumulated onto the membrane surface by immersing the electrode into stirred a 10 mL of 1 μM MB for 2 min without applying any potential. After accumulation of MB, the electrode was rinsed with 5 mM phosphate buffer (pH 7.2) for 30 s to remove the non-specifically bound MB. It was transferred into an analytical buffer added with glucose/substrate in an electrochemical cell for cyclic voltammetry measurements.

2.8. Voltammetric transduction

The current signals of the enzyme reactions were measured using cyclic voltammetry (CV). The scanning potential was measurement from −0.40 to +1.40 V vs Ag|AgCl within scan rate range of 40–150 mV/s in an analytical buffer (5 mM phosphate buffer) with different ranges of pH 6.0–8.0 for optimization. Repetitive measurements were carried out by renewing the surface of the electrode and repeating the same conditions. All experiments were conducted at laboratory temperature condition of 22 ± 2 °C.

3. Results and discussion

3.1. Morphological characteristic of scanning electron microscope

To investigate the properties of this new composite modification electrode made of ESM, ZnONPs and EMIM[Otf], and select the optimum composition for subsequent work. It is an important on the study of electrochemical and morphological structures of two biomaterials are employed as platform for the immobilization of enzyme; egg shell membrane and chitosan. In our experimental works, ESM was chosen as the bio platform for immobilization enzyme. The morphological characteristics of ESM membranes are observed under SEM. In Fig. 1, comparison between the nanostructures of the protein fibers on egg shell membrane (Fig. 1A and B) and the modifications. GOx well dispersed on ESM without addition of nanoparticles shown in Fig. 1C and D. Meanwhile, Fig. 1E and F show particle-like morphology with dense clusters. These morphological structures illustrated ZnONPs had successfully electrodeposited onto the ESM. The ZnONPs appeared as white dots on the protein fiber of ESM. The GOx trapped by the ZnONPs scattering on the fibers. GOx protein molecules were scattered on the whole surface of ZnONPs-[EMIM][Otf] deposited on ESM. When GOx was immobilized on the membrane fibers; the rougher fibers appeared with cluster of lumps of GOx when compare to bare ESM. The fibers of GOx/ZnONPs-[EMIM][Otf]/ESM were found rougher than GOx/ESM modification because it covered with small spherical nanoparticles which increased the surface area of ESM. Meanwhile, chitosan displayed morphological nanostructure (Fig. 2A and B) and particles-like morphology with dense clusters of GOx (Fig. 2C and D). GOx well dispersed on chitosan was observed. The ZnONPs were appeared as white flecks on the fibers. This morphological structure provided evidence that ZnONPs had successfully electrodeposited on the chitosan. GOx protein molecules scattered on the whole surface of ZnONPs-[EMIM][Otf]/ESM. However, chitosan showed less surface area compared to ESM. From the observation, it is concluded that GOx and GOx/ZnONPs-[EMIM][Otf] are successfully immobilized on the ESM for the construction of glucose biosensor. GOx/ZnONPs-[EMIM][Otf]/ESM is better modification

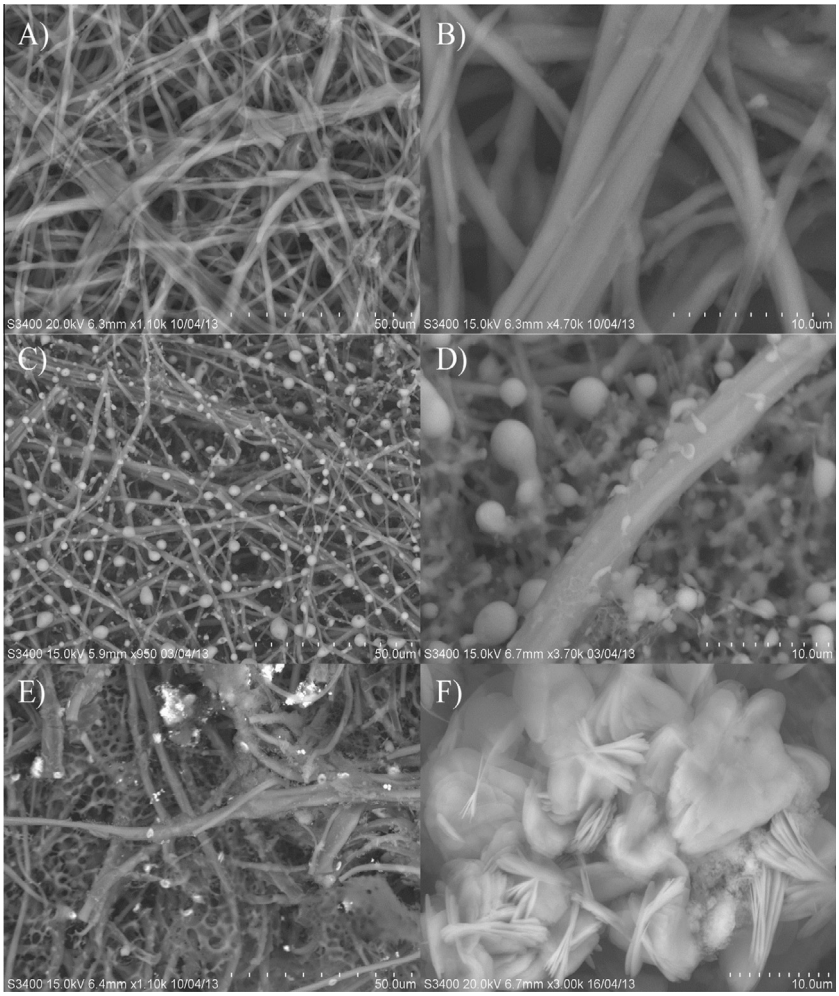


Fig. 1. A scanning electron micrograph of (A) and (B) egg shell membrane [ESM], (C) and (D) egg shell membrane immobilized with glucose oxidase [GOx/ESM] and (E) and (F) egg shell membrane immobilized with ZnO nanoparticles and GOx [GOx/ZnONPs-[EMIM][Otf]/ESM].

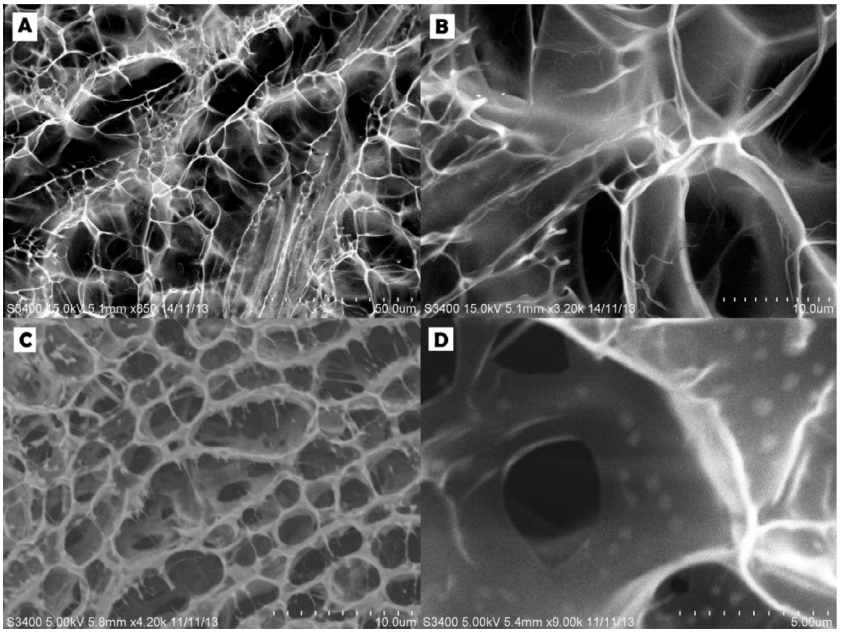


Fig. 2. A scanning electron micrograph of (A) and (B) chitosan (C) and (D) chitosan-nanoparticles immobilized with glucose oxidase [GOx/ZnONPs-[EMIM][Otf]/CHIT].

since it made up of the solid nanostructure of the fibers, use of nanoparticles, ionic liquid which contribute to an increase the surface area of the biomaterial.

3.2. Accumulation of MB

The reduction peak of bare GCE accumulated with methylene blue was increased when compared to bare GCE from -9.05×10^{-6} to -12.77×10^{-6} A. It was showed that the MB was increased with the reduction peak current (Fig. 3). The determination of glucose depends on the electrode modification components and the presence of mediator. Direct electron transfer between enzyme and electrode requires a mediator in order to facilitate the rapid transfer of electrons. The role of the mediator is to shuttle electrons efficiently between the base GCE and the bioactive center of the egg shell membrane [28]. After accumulated MB, the peak current was always increased [27].

3.3. Effects of scan rate

The CV method measured different scan rates using GOx/ESM/GCE in a 50 mM Tris–HCl solution (Fig. 4). The effects of scan rate were found the reduction peak increase with the increasing of scan rate. So, the scan rate of 0.15 V/s gave the highest reduction peak current which was of -10.71×10^{-6} A. From the graph in Fig. 4 shows the effects of the different scan rates. When the increasing scan rate, the anodic and cathodic peak potentials were shifted towards the positive and negative quadrants, indicating charge transfer kinetics limitation. The anodic and cathodic peak currents were increased linearly in proportional of the scan rate on the ranges of 0.04–0.15 V/s. The ratio of cathodic and anodic peak current was in conformance with the characteristics for surface bound redox sites. The overall redox process focused onto the electrode surface that can be considered as the relatively fast on the voltammetry time scale, which is indicated that a surface confined redox process and corresponded to rapid conversion of a surface membrane without diffusion or kinetically controlled reaction step.

3.4. Effects of pH

The effects of pH were measured from 6.0 to 8.5 at interval of 0.5 using a 50 mM Tris–HCl buffer. In Fig. 5 shows the stabilized increase in the product level against pH when electrochemical biosensor was subjected a 0.50 mM of glucose standard with various pH phosphate buffer solutions. The reduction current signal was increased of pH 6.5, after which it was decreased (Fig. 5). The pH maximum sensitivity was found of 6.5 which gave the highest peak value. The immobilized GOx can retain its activity under broad pH conditions, so it is indicating that the membrane and ZnONPs are provided a favorable biocompatible microenvironment for the survival of GOx.

The CV was found the highest peak of -10.00×10^{-6} A when using pH 6.5, while the lowest reduction peak of -6.3×10^{-6} A was recorded during pH 7.5. The optimum pH 6.5 was lower than triglyceride biosensor using egg shell membrane bound enzymes (pH 7.0). Narang et al. showed amperometric sensor based on Prussian blue modified electrodes (pH 8.0) and Pt electrode mounted with PVC membrane bound enzymes (pH 7.5) [28]. In similar, the highest peak current signal was obtained at pH 6.5 when compared to the uses other pH value based on CA membrane bound enzymes [29]. The content of eggshell membrane is a bit more acidic. For that reason the optimum pH was of 6.5.

3.5. Response time

The same concentration of enzyme and substrate were used for the analysis of the response time. The highest current signal was obtained when run the samples until 50 s (Fig. 6). The lowest reduction current signal was obtained for 10 s. The hydrolytic reaction was uncompleted especially when used short time (5 s). The response time of 50 s was found sufficient to convert most of the glucose to gluconic acid and by product hydrogen peroxide.

3.6. Different concentrations detection between eggshell membranes and chitosan

3.6.1. Eggshell membranes

The developed glucose biosensor was conducted using the modified GOx/ZnONPs-[EMIM][Otf]/ESM to react with different concentrations of glucose as shown in Fig. 7. The glucose reaction was completed as like as pH 6.5, scan rate of 0.15 V/s and response time of 50 s, respectively. The developed glucose biosensor was found to be proportional to the glucose different concentration in range of 1.0×10^{-12} to 6 mM. The detection limit was calculated

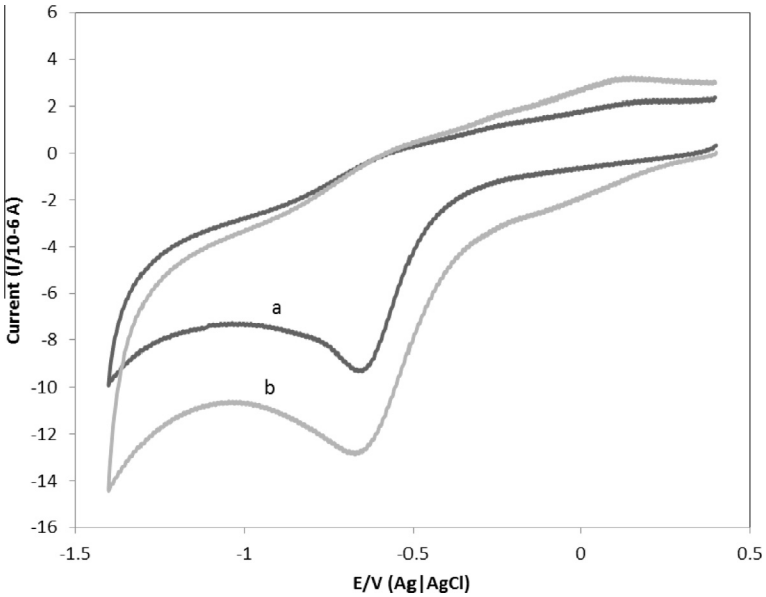


Fig. 3. A cyclic voltammogram of bare electrode with and without soaked in methylene blue for 5 min.

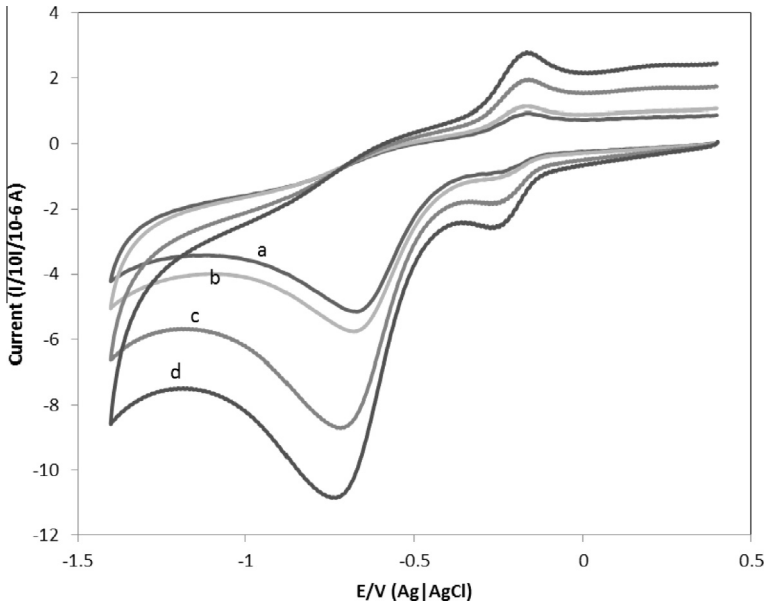


Fig. 4. The cyclic voltammogram of scan rate effects on the detection of conversion of hydrogen peroxide. Scan rate used were: (a) 0.04, (b) 0.05, (c) 0.10 and (d) 0.15 V/s. All were performed at room temperature, 25 ± 1 °C and with same concentration of glucose, 0.5 mM.

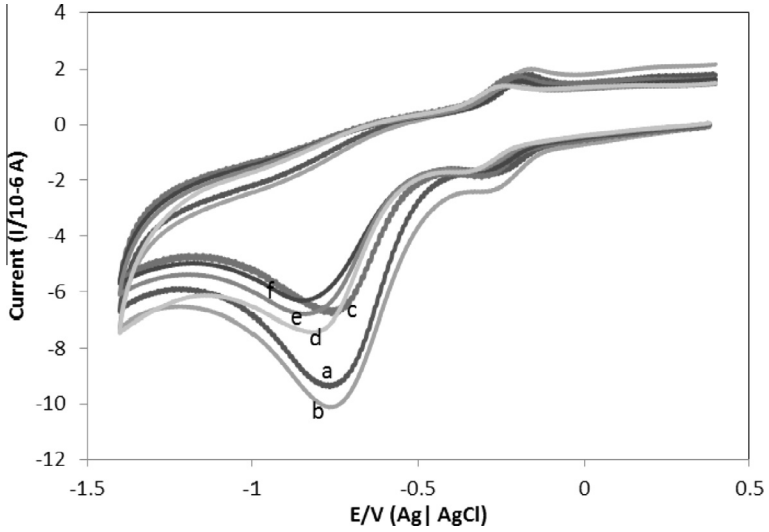


Fig. 5. The cyclic voltammetry of effects of pH of analytical buffer. The pH of buffer used were: (a) 6.0, (b) 6.5, (c) 7.0, (d) 7.5 and (e) 8.0. All were performed at room temperature, 25 ± 1 °C and with same concentration of glucose, 0.5 mM.

to be 1.0×10^{-13} M ($n = 5$). This glucose biosensor found a lower detection limit and a varied linear range as indicated in calibration curve in Fig. 7. The performances of the constructed glucose biosensor and the other electrochemical biosensors based on the nanoparticles and ionic liquid were compared and the results are shown in Table 1.

3.6.2. Chitosan

Chitosan (CHIT) nanocomposite membrane was used in this study due to its low-cost, nontoxicity, and excellent membrane-forming ability. Moreover, chitosan was used as immobilization matrix of the GOx replacing ESM which only detected until glucose at 1×10^{-8} mM (Fig. 8). The surface area of the chitosan was smaller than ESM which had a network-like structure comprising cavities and highly cross-linked protein, so that less ZnONPs could be attached.

Electrochemical response studies were carried out on GOx/ZnONPs-[EMIM][Otf]/ESM bioelectrode for different glucose concentrations in buffer (50 mM, pH 6.5). The peak found at −0.6 V corresponding to the oxidation of H_2O_2 arises due to the enzymatic reaction between glucose oxidase and glucose. The increase in the value of the reduction current with increasing glucose concentration results in the increased concentration of H_2O_2 during the enzymatic reaction. The inset in Fig. 7 shows the variation of oxidation current obtained for the GOx/ZnONPs-[EMIM][Otf]/ESM bioelectrode indicating linearity as 1.0×10^{-12} to 6 mM as well as Fig. 8 for the GOx/ZnONPs-[EMIM][Otf]/CHIT. In this research project used two different type of composite membrane (i) egg shell membrane (ii) chitosan to compare the effectiveness as a good biomaterials for glucose biosensor. By analyzing the variation of peak position as functions of scan rate; it is possible to gain the estimated constants electron transfer rate. The peak shifted because of change of rate constant. For the reactions that are 'slow' (so called *irreversible* electron transfer reactions), the voltage applied will not result in the group of the concentrations at the electrode surface predicted by the Nernst equation [28]. This happens because the kinetics of the reaction is 'slow' and thus the equilibria are not established rapidly (in comparison to the voltage scan rate).

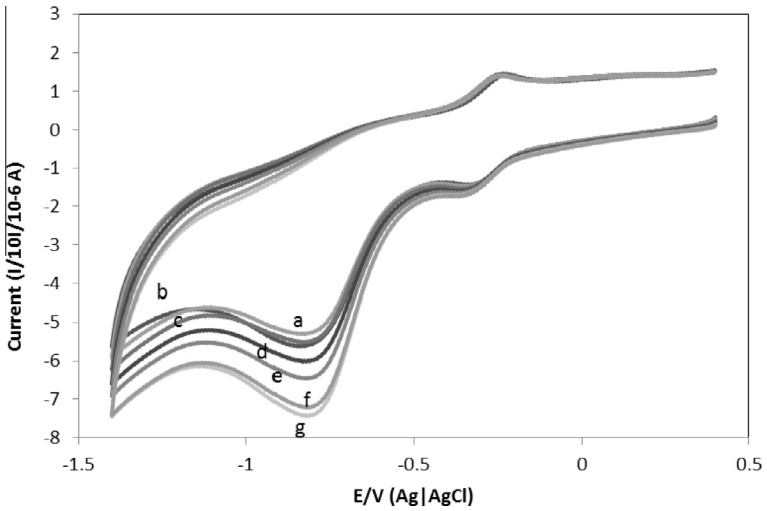


Fig. 6. (A). The cyclic voltammetry of effects of response time for detection of hydrogen peroxide. The response time of biosensor used were: (a) 5, (b) 10, (c) 20, (d) 30, (e) 40, (f) 60 and (g) 50 s. All were performed at room temperature, 25 ± 1 °C and with same concentration of glucose, 0.5 mM.

In some cases of the voltammogram recorded (in which the peak shifted) unlike the reversible reaction present the position of the current maximum −0.6 V shifts depending upon the reduction rate constant (and also the voltage scan rate). This occurs because the current takes more time to respond to the applied voltage than the reversible case.

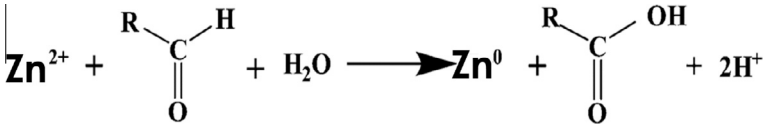
3.7. Immobilization of glucose oxidase

Glucose oxidase (GOx) was initially immobilized onto the surface of fresh eggshell membrane, which was employed to hydrolyze D-glucose with the production of D-gluconic acid and hydrogen peroxide (H_2O_2) as displayed in Eq. (1). Enzyme-immobilized onto egg shell membrane was investigated such as the effects of response time, pH range of buffer, scan rate and glucose concentration on the hydrolysis of glucose.

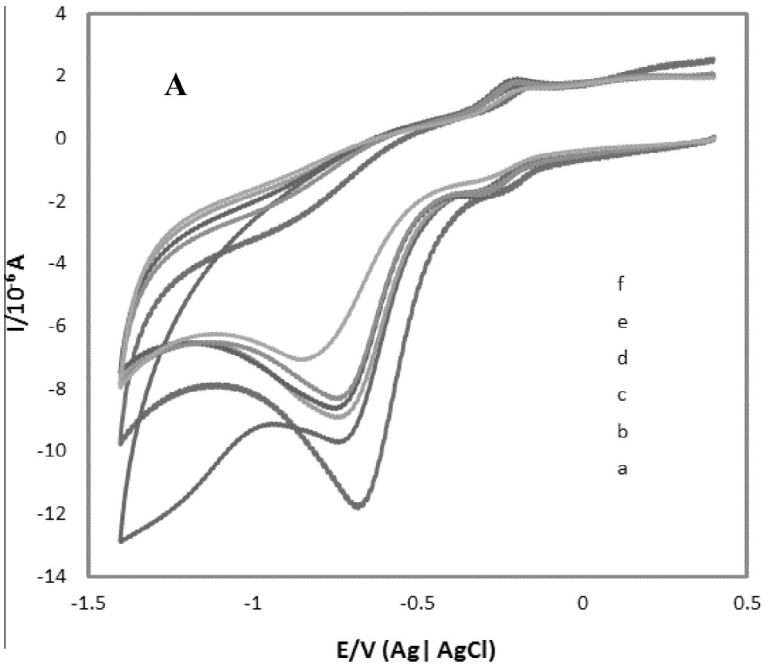
$$\text{D-glucose} + H_2O \xrightarrow[\text{Glucose oxidase}]{} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

Alacam et al. have reported that glutaraldehyde is caused by porous structure of the eggshell membrane and long absorption time enzyme membrane responds during operation [30]. Enzyme biosensor was used of 2.5% (w/w) glutaraldehyde solution as cross-linking agent [5]. Covalent bonding is achieved the immobilization of biological components to a membrane matrix. Cross-linking reaction has same terminal functional groups of the protein and reactive groups on the solid surface of the insoluble bed. So, glutaraldehyde is worked as a cross linking agent between glucose oxidase and egg shell membrane. One –CHO group of glutaraldehyde linked covalently to $–NH_2$ group of egg shell membrane while other –CHO group is covalently linked to $–NH_2$ groups on the surface of glucose oxidase (Scheme 1) [5]. It is provided more stable bioconjugate of enzyme and supported than attained by physical adsorption [31].

The glassy carbon electrode acts as a H_2O_2 transducer. The ESM membrane was attached with glassy carbon electrode for the measurement of byproduct of H_2O_2 [32]. Cyclic voltammetry was the first time used to show the suitability of electrical communication between immobilized enzyme and a glassy carbon electrode via methylene blue incorporated cation film. The generated products were measured by the electrochemical biosensor consisting of GOx/ZnONPs-[EMIM][Otf]/ESM composite mounted on a flow cell. Here, the egg shell membrane was used as a negative charge (anion). The methylene blue mediator is strongly retained inside the membrane through hydrophobic interactions. Due to the some special characterization of ionic liquids such as wide potential windows (a voltage range between which the electrolyte is not oxidize or reduced) and high electrical conductivity, hydrophobicity and the insolubility in water, the extraction and plasticizing ability, they are used in construction of electrochemical biosensors. The reduced GOx donates the excess electron to the [EMIM][Otf]/ZnO-MB nanocomposite matrix to reduce the redox species. The MB reoxidizes by transferring the electron to the external circuit due to efficient electron transfer and good redox property of prepared nanocomposite biomatrix (Scheme 2) [33]. Constructing an electrochemical biosensor that takes advantage of a redox mediator requires only catalytic amounts of the mediator and the enzyme. Due to the electron shuttling, when glucose is added to the solution, the cyclic voltammogram of the mediator has a steady state response [34]. This response by the electrode suggests that the rate of the reaction is limited only by the rate of the glucose oxidation by GOx. The main chemical components of ESM are amino acids (glycine and alanine) and uronic acid [35]. Thereby, there are lots of amino, hydroxyl and carbonyl moieties on the ESM fibers. Among the main ingredients of ESM, uronic acid and saccharides containing aldehyde moieties (R–CHO) can act as reductants to reduce the surface-adsorbed Zn(II) ions to form ZnNPs. The reduction of Zn^{2+} by R–CHO is summarized as follows:



(2)



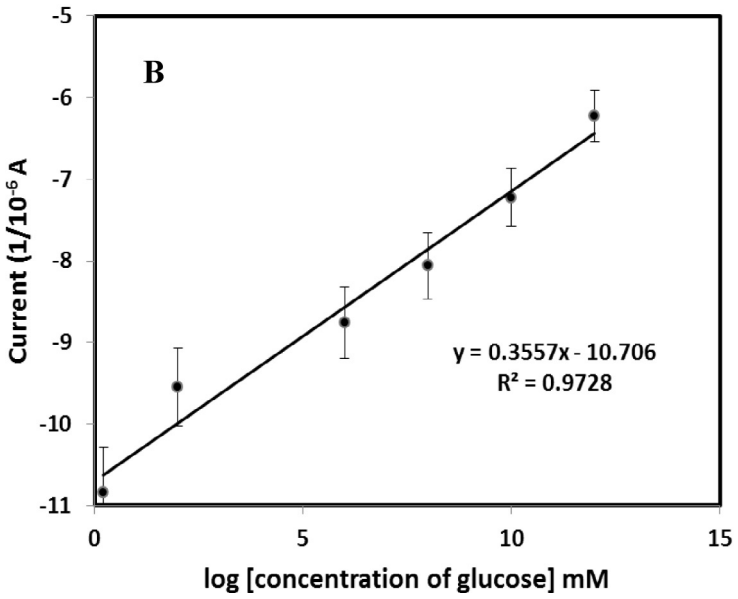


Fig. 7. The CV of different concentration of glucose based on GOx/ZnONPs-IL/ESM was performed at pH 6.5 and temperature of 25 ± 1 °C. The concentrations of glucose (A) used were (a) 0.6, (b) 1×10^{-2}, (c) 1×10^{-6}, (d)1×10^{-8}, (e) 1×10^{-10} and (f) 1×10^{-12} mM. (B) Linear relationship between the reduction peak currents obtained by differential pulse voltammetry and the logarithm of glucose concentrations.

Table 1
Comparison of analytical performance of various glucose biosensors.

References	Range of glucose concentration	Detection limit	Types of electrode used	Modification	Response time
Wu et al. [5]	1.0×10^{-5} to 1.3×10^{-3} mM	1.0×10^{-6} mM	Oxygen electrode	GOx/ESM	100 s
Liu et al. [38]	10–225 μM	5 μM	Oxygen electrode	GOx-PtNPs/ESM	<30 s
Du et al. [39]	5.0×10^{-5} to 1.3×10^{-3} mM	3.50 μM ($S/N = 3$)	Glassy carbon electrode	GOx/AuNPs/CHIT	240 s
Li et al. [15]	1×10^{-6} to 1×10^{-4} mM	5×10^{-7} mM	Colorless glass column	HRP-GOx	60 s
Zhang et al. [40]	16 μM–1.10 mM	8.0 μM	Oxygen electrode	GOx/CHIT	60 s
Zhang et al. [41]	5–25 mM	2.5 mM ($S/N = 3$)	–	AuNPs/GOx/ESM	<60 s
Zheng et al. [42]	0.1–1.0 mM	3.50 μM ($S/N = 3$)	Oxygen electrode	GOx/AuNPs/ESM	<30 s
This work	1.0×10^{-8} to 0.5 mM	1.0×10^{-9} mM	Glassy carbon electrode	GOx/ZnONPs-[EMIM][Otf]/CHIT	50 s
	1.0×10^{-12} to 0.5 mM	1.0×10^{-13} mM	Glassy carbon electrode	GOx/ZnONPs-[EMIM][Otf]/ESM	50 s

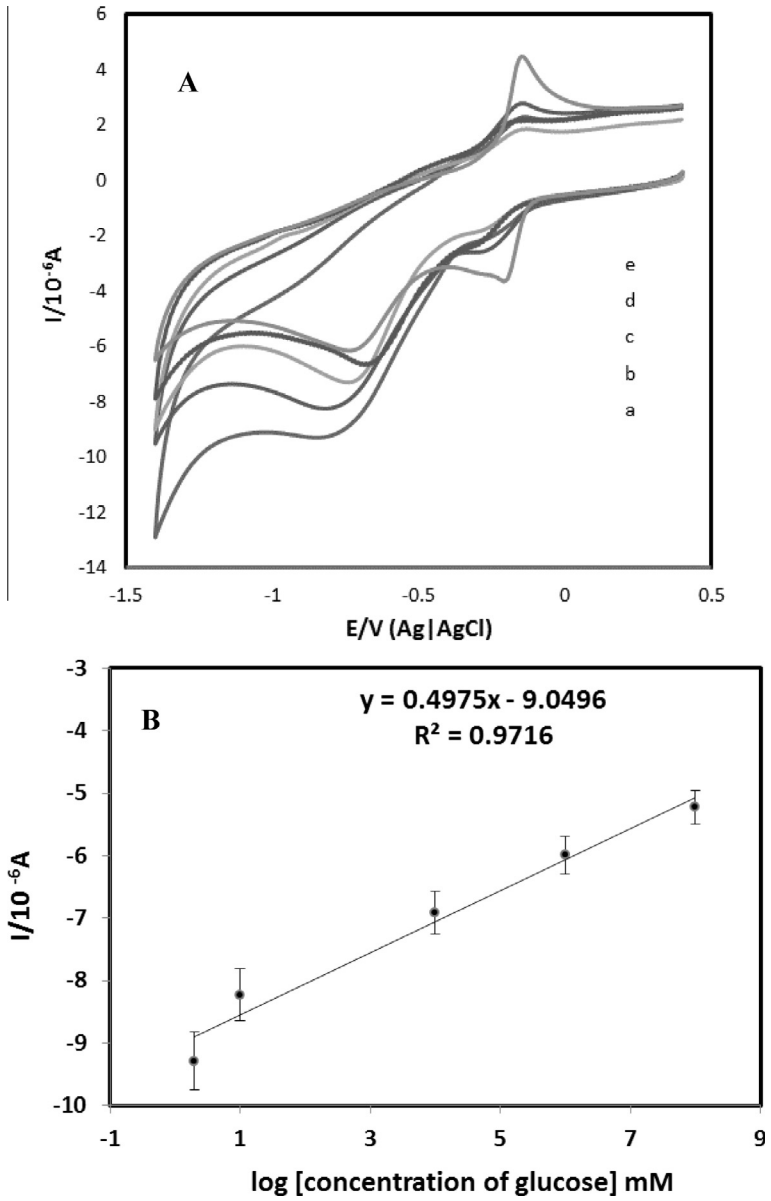
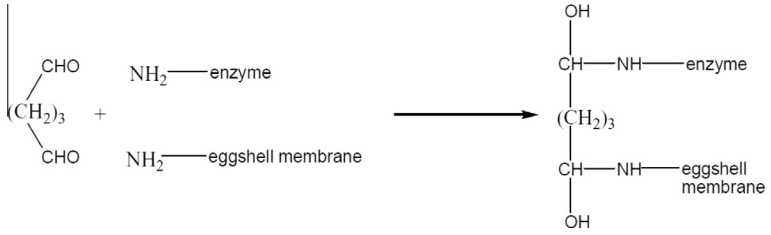


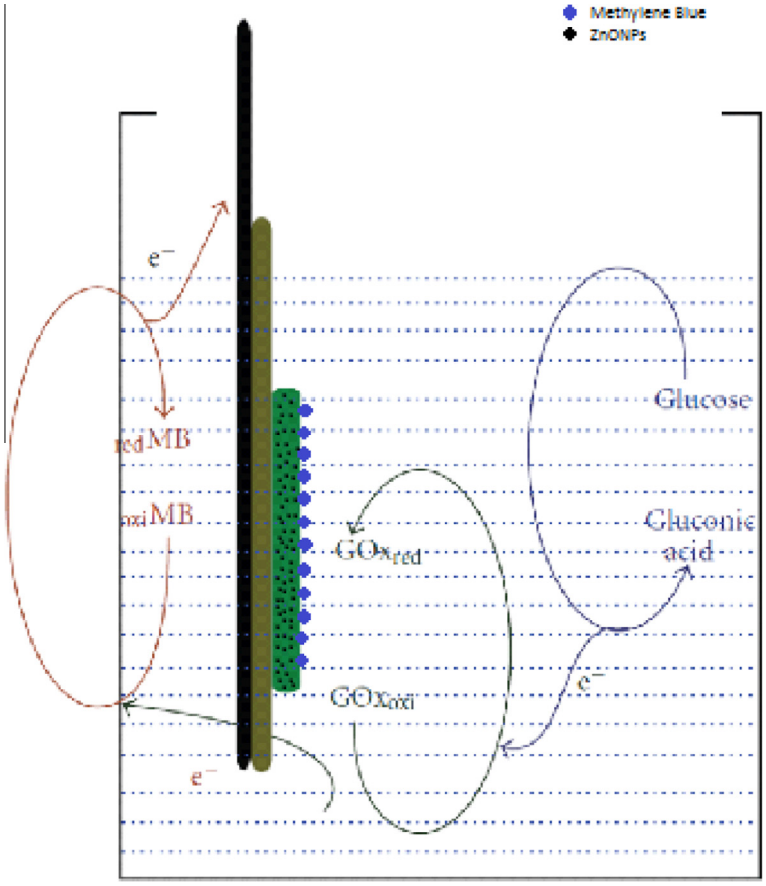
Fig. 8. The CV of different concentration of glucose based on GOx/ZnONPs-IL/CHIT was performed at pH 6.5 and temperature of 25 ± 1 °C. The concentrations of glucose (A) used were: (a) 0.5, (b) 0.1, (c) 1×10^{-4}, (d) 1×10^{-6} and (e) 1×10^{-8} mM. (B) Linear relationship between the reduction peak currents obtained by differential pulse voltammetry and the logarithm of glucose concentrations.



Scheme 1. Reactions of glucose oxidase, glutaraldehyde and protein fiber on egg shell membrane [5].

The Zn(II) ion is initially adsorbed and reduced *in situ* by moieties of the ESM fibers to form Zn(0) atoms which finally deposit on the ESM as ZnONPs. The equation depicts the mechanism interaction between Zn metal towards the membrane used.

Gluconic acid is produced from glucose through a simple dehydrogenation reaction. In 50 s, nearly 100% of the glucose is converted to gluconic acid and hydrogen peroxide in the presence of NAD^+ to facilitate the hydrogen transfer and under optimum condition. The enzyme is induced in the presence of high levels of glucose in the medium, pH 7.0 and elevated oxygen levels. The reduced form of the enzyme is further oxidized by the molecular oxygen, which results in the formation of hydrogen peroxide, a

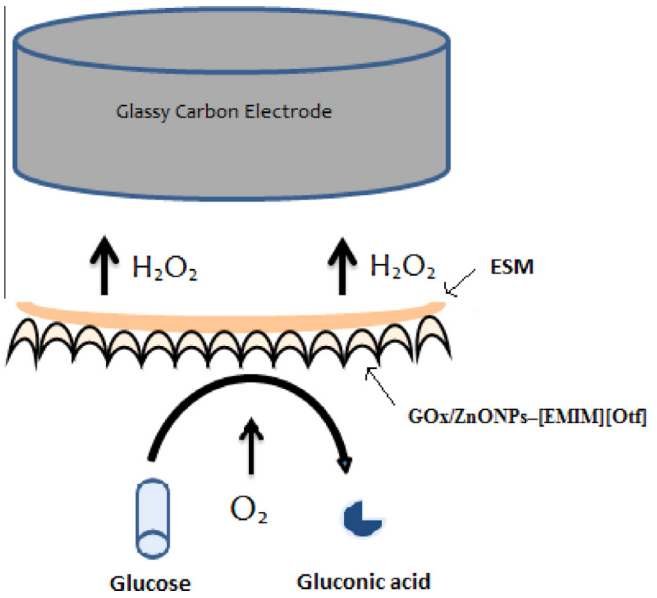


Scheme 2. The electron transfer process of the matrix [33].

by-product in the reaction. Production of gluconic acid is directly linked with the glucose oxidase activity. They are two key parameters which influence the gluconic acid production (i) the molecular oxygen availability and (ii) the pH of the electrolyte. The molecular oxygen in this project was maintained and constant since the aeration rate is fixed. Oxygen was supplied in the form of atmospheric air. Gluconic acid production in enzymatic reaction was influenced by various metal ions such as copper, zinc, magnesium, calcium, iron, etc., [36]. Zn metal ion was used to enhance the catalytic reaction involved.

The direct oxidation of glucose at bare electrodes is not suited for analytical application due to the slow electrode kinetics and high over-potential material, so preparation of modified electrode with catalytic function is practically significance (Scheme 3) [37]. Since, the electro catalytic property of modified electrode is obviously affected by physical and chemical state of the modifiers on electrode surface, designing membrane preparation method is essential. The factors are evaluated by electrochemical techniques based on the affected process of modification and electrochemical property of membrane.

The developed glucose biosensor method has several advantages for determination of glucose earlier methods. Because of it is easily employed covalently bound enzymes on naturally occurring cheaper support membrane (egg shell membrane) which provides stability of immobilized enzyme and better flow of electrons compared to previous membrane electrode [28,29]. Glucose oxidase was immobilized covalently onto egg shell membrane with 100 mg/mL of free enzyme and current signals are measured by using cyclic voltammetry in analytical buffer a 50 mM Tris–HCl buffer (pH 6.5).



Scheme 3. Glucose oxidized into glucose acid at the membrane and O_2 is consumed. H_2O_2 is produced at the same time. Amount of H_2O_2 can be measured by the electrode [37].

4. Conclusions

An novel electrochemical biosensor was developed based on GOx/ZnONPs-[EMIM][Otf]/ ESM/GCE using MB as a redox

indicator. ZnO nanoparticles dispersed onto eggshell membrane and ionic liquid which enhanced the consignment boundary for the immobilization of GOx. The results have indicated that the ZnONPs are provided a nano-sized environment for the intimate precursory interaction between the enzyme and the electrode, which are essential for efficient direct electron transfer. A novel, simple and inexpensive biosensing platform is developed for determination of glucose on the basis of their excellent electrocatalytic activities with glassy carbon electrode towards glucose. The developed glucose biosensor has several advantages over artificial membrane providing a significant increase in electrical conductivity. This study demonstrated that egg shell membrane is a viable alternative for application as a nanocomposite membrane for enzyme immobilization.

Conflict of interest

None declared.

Acknowledgements

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